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ISOLATION AND MOLECULAR DIAGNOSIS OF TRICHODERMA SPP. AND EVALUATION OF ITS EFFECTIVENESS AGAINST DERMATOPHYTES

Waleed Khalid AHMED[1]

Çukurova University, Türkiye

Fatih KÖKSAL

Çukurova University, Türkiye

Abdullah Abdulkreem HASSAN

Tikrit University, Iraq

Melda MERAL OCAL

Çukurova University, Türkiye

Saeed Ullah JAN

Quaid-i-Azam University, Pakistan

Abstract

Dermatophyte infection of the human is difficult to eradicate with drug treatment. An alternative approach to treat dermatophytes might be the application of biological control agents against fungal diseases. Trichoderma spp, were isolated from the soil in Adana province of Turkey. 4 species were identified based on DNA-ITS and ITS4 sequence. T. viride, T. virens, T. harzianum and T. lixii. We tested Trichoderma species against Trichophyton tonsurans and Microsporum canis. All isolates of Trichoderma showed promising results in reducing the growth of pathogens under in vitro conditions, with different concentrations and dual culture assay. However, antagonistic assessment of isolates of Trichoderma against pathogens T.tonsurans and M.canis by concentrations assay (5,10 and 15 ml/L) showed variation in inhibition of pathogens. The colony diameter growth values ranging in all concentrations and isolates from 0.46 to 5.40 cm, The best antifungal concentration was (15 ml/L) with T. harzianum isolate in inhibition for T. tonsurans and M. canis, which reached 0.50 and 0.46 cm respectively. For dual culture assay showed that all isolates of Trichoderma reduced growth T.tonsurans and M.canis. Moreover, the highest inhibition with T. harzianum isolate Which colony diameter reached 1.34 and 1.50 cm, respectively.

Keywords: Trichderma Spp, Molecular Diagnosis, Dermatophytes.

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[1] waleedplant88@gmail.com, https://orcid.org/0000-0002-7659-6336

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Introduction

Dermatophytosis is a common superficial mycotic infection affecting humans widely in the world (Zareshahrabadi et al. 2021). Also are one of the most recurrent human diseases. The diffusion of those infections differ greatly and 10–20% of the world population might be infected with dermatophytes (Hryncewicz-Gwóźdź et al. 2011). The dermatophytes can invade keratinized tissue (skin, hair, nail, feathers and hooves) of humans and animals. Moreover, about 40 species belonging to the genera *Microsporum*, *Trichophyton* and *Epidermophyton* are considered dermatophytes, They possess two substantial properties, keratinophilic and keratinolytic. This means they can digest keratin in vitro in their saprophytic case and utilize it as a substrate, and some might attack tissues in vivo (Kalinowska K et al. 2009). According to their host predilection and natural habitat, dermatophytes are normally grouped in three categories, anthropophilic (human), zoophilic (animal) and geophilic (soil) (Youngchim et al. 2011). Despite the availability of several antifungal drugs for clinical use, it shows considerable drawbacks mainly such as the emergence of drug resistance (Martinez-Rossi et al. 2008). Therefore, it is required to develop new agents with effective modes of action. An alternative approach to the treatment of dermatophytes may be the application of biological control against the pathogens and to reduce to lower inoculum density and activities of the pathogen (Omero et al. 2004).

Biological control is defined as the use of biological processes to aim for lower inoculum density of the pathogen (Baker and Cook 1974). Antagonistic interactions between microorganisms various in their nature and include antibiosis, parasitism, lysis, and competition. Understanding the mechanisms associatedwith the biocontrol process is considered to be a key factor in developing beneficial biocontrol agents. Using a parasitic fungus as the biocontrol agent might term mycoparasitism (Cook and Baker 1983). *Trichoderma* spp. is well-known to control a wide range of economically important plant pathogenic fungi (Estrella and Chet 1998). Differ types of *Trichoderma* have been reported to successfully inhibit pathogen growth and evolution (Benhamou and Chet 1993). The capacity of *Trichoderma spp*. to invade fungal pathogens at various stages of their development led to the realization of their application as strong biocontrol agents (Chet 1987). According to the finding some *Trichoderma spp*. can be producing antibiotics or extracellular lytic enzymes (Elad et al. 1982). control soil-borne fungal pathogens, Certain airborne pathogens (Chilosi et al. 2020) and Dermatophytes pathogens (Aasi and Al-Aaraji 2018; Al-Obaidy and Al-Rijabo 2011; Omero et al. 2004).

The objective of this study was the molecular diagnosis of *Trichoderma spp* isolates and evaluating the antagonistic activity against *T.tonsurans* and *M. canis*.

Materials and Methods

Isolation and Molecular identification of *Trichoderma spp*

The samples were collected from the soil. 5-fold serial dilutions of each soil sample were prepared . 5 gr was taken from the soil and add to 100 ml distilled water and added 0.5 ml to the potato dextrose agar medium (PDA). The inoculated PDA plateswere incubated at 28°C for 120 h. Then colonies appearing on the plates were purified.For molecular identification, nucleotides sequences of the internal transcribed spacer (ITS) region were analyzed. Genomic DNA was extracted from mycelia of *Trichoderma* isolates using Chelex®100 Resin Kit (BioRad Laboratories, USA) according to the manufacturer's instructions.

PCR Amplification of ITS gene and sequencing

The primer pair ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC) (Integrated DNA Technologies company, USA) using for amplification of ıts gene.PCR amplification was performed in a total volume of 25µl containing. the mixture contained 5 µL of master mix, primers 1µL of the forward, and 1µL of the reverse, 16 µL of double-distilled H_2O and 2 µL of DNA. programmed as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 1min, 58°C for 1 min and 72 °C for 1 min, with a final extension at 72 °C for 7 min. PCR products were separated on 1.5 % agarose gels containing ethidium bromide and visualized under UV light. The PCR products were used as templates for the sequence analysis of the internal transcribed spacer (ITS) 1 and 4 of DNA. The PCR products were cleaned up by adding 2.5 µl of GML ExoSAP (GML, Switzerland) to each 5 µl amplicon. Sequencing of both strands of the PCR product was performed on an ABI37 sequencing instrument according to the protocol supplied by the manufacturer using the Big Dye™Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA). BLAST program was used to compare the ITS gene sequence of the test bio-agents against the nucleotide collection database.

Preparation Filtrate of *Trichoderma spp*

For testing the antifungal activity of *Trichoderma* isolates, three mycelial agar plugs randomly were taken from the fungal colony grown on PDA (4 day old), plugs were inoculated in 100 ml potato dextrose broth (PDB) in a 250-ml Erlenmeyer flask, in shake culture (150 rpm) at room temperature for 15 days. Cultural filtrates were obtained by centrifuging the cultures at 12,000 rpm and filtering through 0.22 µm millipore filters to remove hyphal fragments and were stored at 4°C until use.

Antifungal assay

Trichoderma spp filtrate (5,10 and 15 ml) were mixed separately with 100 ml of SDA medium, then pouring the culture medium into the Petri dishes after that the dishes were inoculate with pathogenic fungi *T. tonsurans* and *M.canis* then incubated at 28°C. The results were taken after the pathogenic fungi covered the entire area of the dish in the control treatment.

Dual cultural screening of antagonistic fungi

Antifungal activity of each *Trichoderma* isolates against the pathogenic fungi *T. tonsurans* and *M.canis* were tested in dual cultures on SDA. As *Trichophyton* grows much slower than *Trichoderma* its homogenate was spread over two-thirds of SDA Petri dish and incubated for 2 days at 28°C. *Trichoderma* hyphae disc was then placed over the empty third of the Petri dish. Antagonism was identified by overgrowth of *Trichoderma spp.* on *Trichophyton spp.* within 7 days from inoculation. *Trichoderma spp.* overgrowth was quantified as the distance of overgrowth (cm) (El-Naggar et al. 2008).

Statistical analysis

The experiment was carried out according to the design (C.R.D) under in vitro conditions and data were analyzed for variance (ANOVA) using the SAS software version (9.1.) The mean were compared with the least significant difference (L.S.D) at a level of 0.05.

Result and Discussion

The comparison of *Trichoderma spp* sequences (the sequence of the isolates A, S, H and ASH) with the NCBI sequences database allowed the identification of isolates as the *T.*

Viride T. virens T. harzianum and *T. lixii* respectively.The similarity percentage for all isolates reaches 100 % A summary of the sequence results is presented in Fig 1.

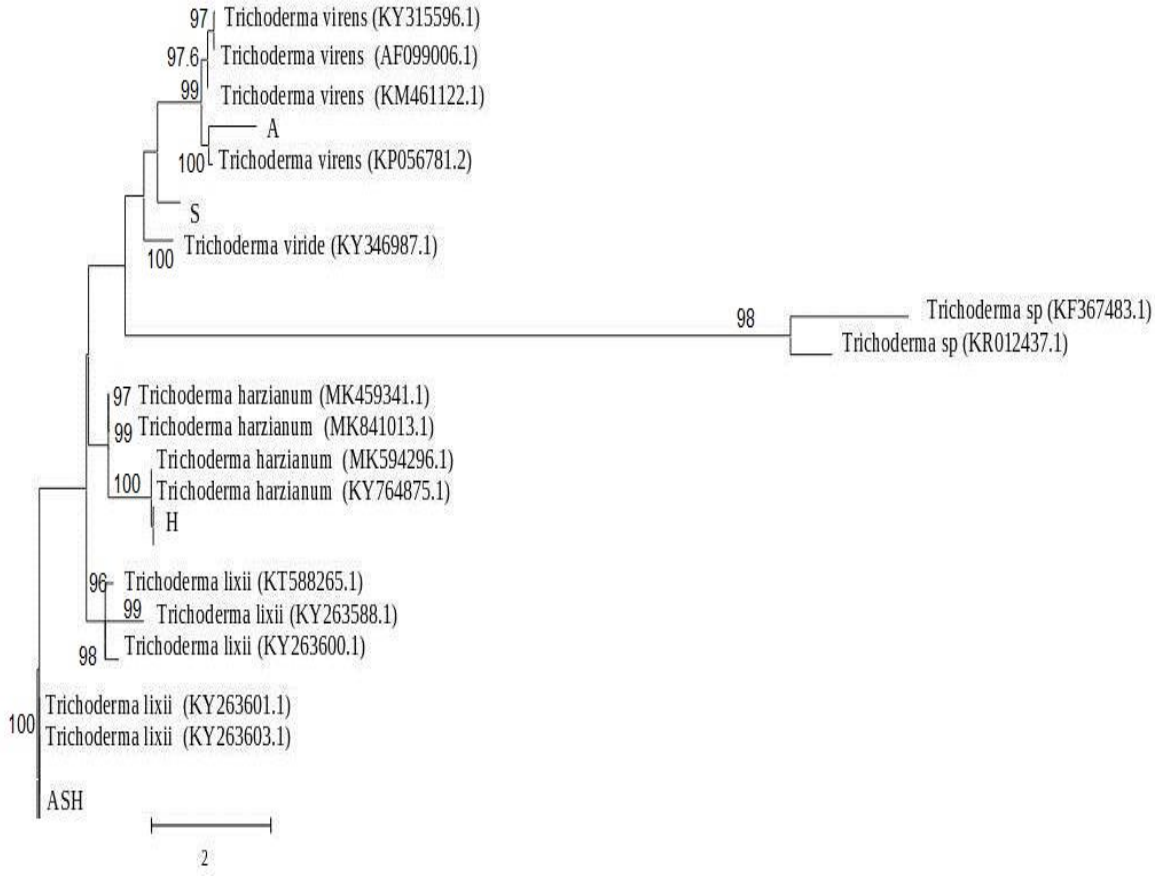

Fig.1.Phylogenetic tree based on internal transcribed spacer (ITS) sequences of the selected Trichoderma isolates. The tree was generated from the ITS sequences of *Trichoderma* isolates by the neighbor-joining method using MEGA10 (http://www/megasoftware.net/).

Effect of the filtrates of *Trichoderma* isolates in the growth inhibition of *T.tonsurans* and *M. canis*.

Results showed in table 1. that all the *Trichoderma* isolates carried out inhibition activity against *T. tonsurans* and *M. canis*. The growth inhibition of pathogens increased with increasing of filtrates concentrations. The highest value was *T. harzianum* treatment in 15 ml/L concentration, the Colony diameter reached 0.5 and 0.46 cm respectively, compared to the control (pathogens alone) 9.00 cm. This inhibition might be due to antibiosis, nutrient competition or cell wall degrading enzymes, or the extract contains active pharmacological compound can be responsible for the rate of inhibition. This results in agreement with (Al-Obaidy and Al-Rijabo 2011). *Trichoderma spp* have resulted as effective antagonists inhibiting the growth of pathogenic dermatophyte fungi, the results being similar to those shown by (Omero et al. 2004)

Table 1. Effect of the filtrates of *Trichoderma* isolates in the growth inhibition of *T.tonsurans* and *M. canis*.

	Colony diameter(cm)					
	T.tonsurans			***M. canis***		
Isolates	5 ml/L	10 ml/L	15 ml/L	5 ml/L	10 ml/L	15 ml/L
T. viride	4.67	3.86	1.03	5.40	3.33	0.86
T. virens	4.17	4.10	0.83	4.80	3.40	0. 80
T. harzianum	4.33	3.6	0.50	4.56	3.13	0.46
T. lixii	4.73	3.93	0.90	5.06	3.90	0.93
Control	8.00	8.00	8.00	8.00	8.00	8.00
L.S.D P<0.05	0.73	0.97	0.66	0.84	0.68	0.40

The mean inhibition percentage of *T.tonsurans* and *M canis* by different *Trichoderma* isolates in Dual culture .

According to the results among all *Trichoderma* isolates superior on inhibition of *T.tonsurans* and *M canis*compared with the pathogens alone, the highest value *T. harzianum* of the pathogenic fungus with both pathogens, the inhibition reached 1.43 and 1.50 cm respectively, compared to 7. 50 cm, in the presence of the pathogens alone. The dual culture of *Trichoderma* species and Pathogens led to dermatophytes growth was in general inhibited, like as reactions have been reported on other pathogens (Hanson and Howell 2004). Mycoparasitism behavior displayed by *Trichoderma* species in interaction regions between dermatophytes and *T. viride, T. virens, T. harzianum* and *T. lixii*. The mycelia of *Trichoderma* strains grew on the surface of the pathogens always roll around their mycelia and then break through their cell walls immediately, may the action of the enzymes lead to disintegrating mycelia of pathogens (Padmodaya and Reddy 1996; Omero et al. 2004).

Table 2. Antagonism average of two dermatophytes strains with four *Trichoderma* species in Dual culture .

	Colony diameter(cm)	
isolates	***T.tonsurans***	***M canis***
T. viride	2.33	1.66
T. virens	2.00	2.33
T. harzianum	1.34	1.50
T. lixii	1.50	2.00
Control	7.50	7. 50
L.S.D P<0.05	1.12	1.09

Trichoderma spp. is known for its potential as a biocontrol against plant pathogenic fungi. The direct mycoparasitic activity of fungi of the genus *Trichoderma* has been proposed as one of the major mechanisms involved in their antagonistic activity against phytopathogenic fungi and dermatophytes fungus as in nail, skin, and hair infections (Chet 1990; Omero et al. 2004) A crude extract concentration and dual culture test showed that *Trichoderma* species antagonistic to the dermatophytes, *T.tonsurans* and *M.canis* (Table 1

and Table 2), *T. harzianum* were found to be most effective among the species tested in this study. This new antagonism might lead to its application as a perfect viable mycoparasitic organism or using the accompanying secreted products such as hydrolytic enzymes or antibiotic compounds to affect lysis and growth arres(Peterbauer et al. 1996; Chet 1990). Although production of secreted compounds has been showed for *Trichoderma spp*. grown in synthetic poor medium without induction by *Trichoderma* hyphae, significant antibiotic potential has been showing in accord with previous study for other targeted fungi (Howell et al. 1993) and for the first time for *T.tonsurans* and *M.canis*. The results indicate that *Trichoderma* spp. are a potential antifungal biocontrol agent against dermatophytes can be used alone or with other chemical antibiotics as a treatment to eliminate dermatophytes that infect human.

Conclusion

The filtrates concentrations of Trichoderma isolates showed a high antifungal activity against T.tonsurans and M. canis . The filtrates from Trichoderma isolates can be used as an effective treatment to eliminate dermatophytes rather than the use of chemical antibiotics.

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